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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

SHP-PT059

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/355555

INTERNATIONAL APPLICATION NO.
PCT/GB98/03686INTERNATIONAL FILING DATE
4 December 1998PRIORITY DATE CLAIMED
4 December 1997

TITLE OF INVENTION

HLA-E BINDING

APPLICANT(S) FOR DO/EO/US Braud et al.

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
 - International Search Report (included with International Publication);
 - International Preliminary Examination Report; and
 - Certificate of Mailing by Express Mail.

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)

Not Yet Known

INTERNATIONAL APPLICATION NO
PCT/GB98/03686ATTORNEY'S DOCKET NUMBER
SHP-PT05917. ☒ The following fees are submitted:**BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :**

Neither international preliminary examination fee (37 CFR 1.482)
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO
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International preliminary examination fee (37 CFR 1.482) not paid to
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International preliminary examination fee paid to USPTO (37 CFR 1.482)
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\$ -

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	29 - 20 =	9	X \$18.00
Independent claims	9 - 3 =	6	X \$78.00

\$ 162

\$ 468

MULTIPLE DEPENDENT CLAIM(S) (if applicable) 0 + \$260.00

\$ 0

TOTAL OF ABOVE CALCULATIONS =

\$ 1,470

Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement
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SUBTOTAL =

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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C. Frederick Koenig III, Esquire

NAME

29,662

REGISTRATION NUMBER

PTO/SB/1 (12-87)
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STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) & 1.27(d))—NONPROFIT ORGANIZATION		Docket Number (Optional) SHP-PT058
Applicant, Patentee, or Identifier: <u>Braud et al.</u>		
Application or Patent No.: <u>09/555,555</u>		
Filed or issued: <u>Not Yet Known</u>		
Title: <u>HLA-E BINDING</u>		
I hereby state that I am an official empowered to act on behalf of the nonprofit organization identified below:		
NAME OF NONPROFIT ORGANIZATION <u>Isis Innovation Limited of Oxford University</u>		
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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))		
NAME OF PERSON SIGNING <u>Dr. T. Cook</u>		
TITLE IN ORGANIZATION OF PERSON SIGNING <u>Managing Director</u>		
ADDRESS OF PERSON SIGNING <u>Ewert House Ewert Place Summertown, Oxford</u> <u>OX2 7BZ UK</u>		
SIGNATURE <u>T. Cook</u> DATE <u>28-7-00</u>		

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09/555555

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422 Rec'd PCT/PTO 01 JUN 2000 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the **PATENT APPLICATION** of:

Braud et al.

Application No.: Not Yet Known

Our File: SHP-PT059

Filed: Not Yet Known

Date: June 1, 2000

For: HLA-E BINDING

Group: Not Yet Known

Examiner: Not Yet Known

PRELIMINARY AMENDMENT

Box PCT

Assistant Commissioner for Patents

Washington, D.C. 20231

Sir:

Prior to examination, please amend the application as follows.

IN THE ABSTRACT

The Abstract has been reproduced and is submitted herewith on a separate sheet numbered page 40.

IN THE CLAIMS

In claim 8, line 1, please delete "any one of claims 5 to 7" and insert therefor --claim 5--.

In claim 13, line 1, please delete "any one of claims 9 to 12" and insert therefor --claim 9--.

In claim 14, line 2, please delete "or claim 4".

In claim 16, line 2, please delete "or claim 15".

In claim 17, line 4, please delete "or claim 4, or claim 8,".

In claim 23, lines 1-2, please delete "any one of claims 20 to 22" and insert therefor --claim 20--.

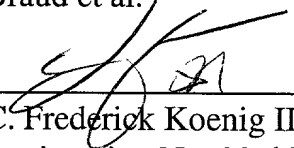
In claim 28, line 2, please delete "any one of claims 24 to 26" and insert therefor --claim 24--.

REMARKS

The Abstract has been reproduced on a separate sheet in accordance with U.S. practice. Claims 8, 13, 14, 16, 17, 23 and 28 have been amended to delete reference to multiple claims. Early consideration and allowance of pending claims 1-29, as amended, is respectfully requested.

Respectfully submitted,

Braud et al.

By 
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CFK/ras
Enclosure

HLA-E BINDING

This invention relates to methods of identifying, targeting and of isolating a group of CD94+ cells, in particular a group of CD94+ cells including natural killer (NK) cells and a subset of T cells. The invention also relates to methods of targeting functional moieties such as toxins to the CD94+ cells. The invention further relates to multimeric complexes of HLA-E for use in the methods.

Human leukocyte antigen-E (HLA-E) is a nonclassical MHC class Ib molecule of very limited polymorphism. Human nonclassical MHC class Ib molecules (which include HLA-E, HLA-F and HLA-G) are homologous to classical MHC class Ia molecules (HLA-A, HLA-B and HLA-C) but are characterised by limited polymorphism and low cell surface expression (reviewed in Shawar et al 1994 *Annu. Rev. Immunol.* 12: 839). The mouse MHC class Ib molecule Qa-1 shares some characteristics with HLA-E in that it displays a broad tissue distribution and some structural similarities in the peptide-binding groove (reviewed in Soloski et al 1995 *Immunol. Rev.* 147: 67).

Whereas the function of the classical MHC class I molecules in presenting peptides derived from cytosolic proteins to CD8+ T cells is well-established, the function of nonclassical MHC molecules remains unknown, in particular for HLA-E. HLA-E is transcribed in most tissues. Recently, we have shown that HLA-E is able to bind in its peptide-binding site peptides derived from MHC class I leader sequences at positions 3 to 11 (Braud et al 1997 *Eur. J. Immunol.* 27: 1164-1169). The optimum binding peptide is a nonamer. Using alanine and glycine substitutions, it was established that there are primary anchor residues at positions 2 and 9 of the peptide and secondary anchor residues at position 7 and possibly position 3. The literature suggests that HLA-E is localised in the

endoplasmic reticulum (ER) and might have a role in the loading of peptides onto classical MHC class I molecules in a similar way to HLA-DM for MHC class II molecules. Neither mouse cells transfected with HLA-E and human $\beta 2$ microglobulin ($\beta 2m$) nor the 721.221 cell line which only
5 expresses HLA-E and HLA-F, show surface expression of HLA-E (Ulbrecht et al, *J. Immunol.* 1992 149: 2945 - 2953 and *J. Exp. Med.* 1992 176: 1083 - 1090).

Assembly of MHC class I molecules occurs in the endoplasmic reticulum (ER) and requires peptide translocation through the
10 transporter associated with antigen processing (TAP) (reviewed in Cerundolo et al 1996 In *HLA and MHC genes, molecules and function*, Edited by Browning M, McMichael A. Oxford: Bios. Scientific Publisher Ltd; 193 - 223). In human cells, newly synthesised MHC class I heavy chains associate with calnexin which is later displaced by the association of $\beta 2m$.
15 Following dissociation of calnexin, class I- $\beta 2m$ heterodimers are stably associated with another ER resident protein, calreticulin. Another molecule, tapasin, which is associated with TAP and with MHC class I-calreticulin complexes, acts as a bridge between them. MHC class I association with TAP facilitates peptide binding and the class I molecules
20 are released and exported to the cell surface upon stable loading of peptide.

Natural killer (NK) cells are cytotoxic cells which have the morphology of large granular lymphocytes and are normally defined by their activity. They use recognition systems which are not yet clearly
25 understood. Recognition of tumour cell lines and virally-infected cells is however driven by the absence of MHC class I at the target cell surface (some MHC class I molecules interact with specific NK receptors). NK cell cytotoxicity is mediated by the interaction between Fas and Fas ligand or by the release of the contents of the intracellular granules including the

pore forming protein perforin and the serine protease granzyme B. NK cells are generally but not exclusively CD3- and CD56+. They may also be CD16+ and some are also CD8+. Certain CD8+ T cells have an NK cell-like function, in that they are able to kill MHC class I negative cells.

5 NK cells express receptors that interact with MHC class I and serve to inhibit or activate NK cell-mediated cytotoxicity. The killer cell immunoglobulin-like receptors (KIR), which are members of the immunoglobulin superfamily, make up one such group of receptors.

Another NK cell inhibitory receptor which has a similar effect
10 is the CD94/NKG2A receptor from the C-type lectin superfamily, which is expressed at the cell surface as a heterodimer of CD94 covalently associated with NKG2A. CD94 also associates with other members of the NKG2 family, which consists of four closely related molecules NKG2A, B, C and E and two more distantly related molecules NKG2D and F.
15 CD94/NKG2A and B are both inhibitory NK cell receptors which interact with MHC class I to inhibit NK cell lysis, while CD94/NKG2C is a stimulatory NK cell receptor which interacts with MHC class I to perform an NK cell triggering function.

The CD94/NKG2C activator receptor contains a third subunit
20 DAP12, which is expressed as a disulphide-bonded homodimer and interacts with NKG2C via charged residues in the transmembrane domains (Lanier, 1998, *Nature* 391:703; Lanier et al, 1998, *Immunity* 8:693). DAP12 is necessary for efficient transport of the CD94/NKG2C complex to the cell surface. Ligation of CD94 on CD94/NKG2C/DAP12 transfectants
25 causes tyrosine phosphorylation of DAP12, suggesting that it induces cellular activation via DAP12.

Four genes encode the NKG2 glycoproteins: NKG2A, NKG2C, NKG2E and NKG2D/F (Houchins et al, 1991, *J. Exp. Med.* 173: 1017; Plougastel et al, 1997, *Eur. J. Immunol.* 27: 2835). NKG2A and B
30 are alternative splicing products (differing by an 18 amino acid segment

immediately outside the transmembrane region). NKG2C is highly homologous to NKG2A and B with 94% homology in the external C terminal domain and 56% homology through the internal and transmembrane regions. NKG2D is distantly but significantly related (21%
5 homology) as too is NKG2F.

It has recently been discovered that HLA-E is stably expressed at a low level on the surface of cells. Its expression at the surface correlates with co-expression of human MHC class I molecules which possess a peptide in their leader sequence capable of binding to
10 HLA-E. Loading of these signal sequence-derived peptides is TAP and Tapasin-dependent and HLA-E assembly appears to be similar to classical MHC class I assembly (Braud et al 1998 *Current Biology* 8:1-10).

It has now also been discovered that HLA-E binds to NK cells expressing receptors CD94/NKG2A, B and C. The majority of NK cells
15 express CD94/NKG2 receptors and the majority of NK cells are capable of binding HLA-E. HLA-E also binds to a small subset of T cells expressing CD94/NKG2 heterodimers.

Furthermore, surface expression of HLA-E provides protection against killing by CD94/NKG2A+ NK cells.

20 In addition, it has been discovered that multimeric HLA-E molecules bind strongly to NK cells and the T cell subset expressing CD94/NKG2.

In its broadest sense, the invention provides the use of the interaction between HLA-E and NK cells and/or a subset of T cells, to
25 identify and/or target and/or isolate those cells; and HLA-E in a suitable form for such use.

The invention provides in one aspect a method of causing an interaction of CD94/NKG2+ cells, which method comprises contacting the cells with HLA-E under binding conditions.

30 The invention thus encompasses in one embodiment a

method of identifying the presence of CD94/NKG2+ NK cells and T cells in a sample, which method comprises contacting the sample with HLA-E under suitable binding conditions and detecting binding of HLA-E to the cells.

5 In another embodiment, the method according to the invention provides a method of selecting for CD94/NKG2+ cells, in particular NK cells and a subset of T cells, from a sample, which method comprises contacting the sample with HLA-E under binding conditions and separating cells bound to the HLA-E from the mixture.

10 In still another embodiment there is provided a method of killing or inactivating NK cells and a subset of T cells, which method comprises contacting the cells with HLA-E under binding conditions and carrying out targeted killing on the bound cells. Any targeted killing method may be used, for example NK cells may be identified by detecting bound
15 HLA-E, and then destroyed by use of a laser, or the HLA-E may carry a toxic moiety which kills or inactivates the cells to which the HLA-E binds.

In yet another embodiment, the invention provides a method of modifying NK cell activity against a potential target cell, by expressing HLA-E at the surface of the target cell. In this embodiment, the binding of
20 the CD94/NKG2 receptors to the cell surface HLA-E thus causes an interaction between the CD94/NKG2+ cells and the HLA-E bearing cells. In the case of CD94/NKG2A or B receptors, this will be an inhibitory interaction the effect of which is to protect the HLA-E bearing cell from killing by the NK cells. In the case of CD94/NKG2C receptors there may
25 be an NK cell stimulatory effect. In the case of NK cells expressing both inhibitory and activator CD94/NKG2 receptors, the overall effect of an interaction with HLA-E at the target cell surface is an inhibitory one, since the inhibitory receptors override the stimulatory receptors.

Thus, the invention involves the use of the newly discovered
30 HLA-E - CD94/NKG2 receptor binding partnership for a variety of possible

purposes. The interaction in the method according to the invention may be simply the binding of the CD94/NKG2 receptors to the HLA-E.

Alternatively or additionally the interaction of the CD94/NKG2+ cells with the HLA-E may give rise to an effect on the activity of the CD94/NKG2+

5 cells, such as an inhibitory effect

In further aspects, the invention provides CD94/NKG2+ cells isolated by the method according to the invention; and a population of cells depleted of CD94/NKG2+ cells by the method according to the invention.

In another aspect the invention provides a non-human
10 mammalian cell which expresses HLA-E at the cell surface by virtue of a nucleic acid encoding HLA-E integrated into the genome of the cell. The nucleic acid encoding HLA-E is a heterologous nucleic acid in the sense that it is not found in those cells in nature. The invention also provides recombinant animals comprising such cells, which animals include
15 transgenic animals which contain HLA-E-encoding nucleic acid material in their somatic and germ cells, as well as animals which are recipients of a transplant from such transgenic animals .

In another aspect the invention provides a method of testing a compound for biological activity, which method comprises:

- 20 (i) providing cells expressing CD94/NKG2 receptors at the cell surface;
- (ii) contacting the cells with HLA-E in the presence of the test compound; and
- (iii) determining whether the presence of the compound affects
25 the binding of HLA-E to the cells;

and compounds identified by the method as being compounds which affect the binding of HLA-E to CD94/NKG2 receptors.

Preferably the cells expressing CD94/NKG2 receptors in the method according to this aspect of the invention do not naturally express
30 the receptors, and most preferably they are non-human cells. The cells are

preferably stable transfectants, that is to say they contain nucleic acid material expressing CD94/NKG2 stably integrated into their genome.

Compounds such as antibodies, in particular monoclonal antibodies, may be screened by the method for a particular desired property. Compounds may be identified by the method which interfere specifically with the interaction between HLA-E and CD94/NKG2A but not CD94/NKG2C, or vice versa. Antibodies with such specificity will be useful, for example to enable CD94/NKG2A NK cells to be distinguished from CD94/NKG2C cells. Therapeutic uses for antibodies which inhibit the binding of HLA-E to CD94/NKG2 receptors are also envisaged, for example in bone marrow transplantation. It can be difficult to find a matched human donor and allogeneic cells might not possess MHC class I ligands that can engage the inhibitory receptors of all NK cells. An antibody which specifically blocks the binding of NK cells to the activator receptor CD94/NKG2C will therefore be useful. Another example of a possible therapeutic use of the antibodies is in the treatment of certain autoimmune diseases.

In another aspect the invention provides a multimer of HLA-E comprising two or more HLA-E molecules, said multimer having enhanced binding capability compared to non-multimeric HLA-E, optionally labelled with a signal moiety. The multimers of HLA-E contain at least two subunits which have the binding properties of HLA-E receptors (that is they bind CD94/NKG2 receptors), linked together to produce a bi-functional or multifunctional species. Each subunit comprises all or a substantial part of the extracellular region of HLA-E, generally at least the $\alpha 1$, $\alpha 2$ and $\alpha 3$ HLA-E domains, together with $\beta 2$ microglobulin, and a suitable peptide in the peptide binding groove. Preferred HLA-E multimers are tetramers, but other multimers for example dimers, trimers and multimers containing 5, 6, 7 etc. HLA-E molecules are not excluded.

In another aspect the invention provides a recombinant HLA-

E coupled to a toxic agent. The purpose of the toxic agent is to kill or inactivate cells to which the recombinant HLA-E binds. It is sufficiently toxic for that purpose. The toxic agent preferably has a localised effect, that is it preferably does not affect surrounding cells to which the HLA-E is not bound. Preferably the recombinant HLA-E is in the form of a multimer.

The invention is useful for diagnostic purposes and in general for monitoring diseases. Detection and/or quantitation of NK cells or a sub-population of NK cells, or a sub-population of T cells (which may be further identified by co-staining with anti-CD8 or anti-CD4 antibody or antibodies or ligands to other T cell markers) will be useful in a variety of conditions, including the following:

(i) Cancer, Lymphomas and Leukaemias (particularly large granular cell Leukaemias).

NK cells are believed to have an anti-tumour cell activity. A marker for progress of therapy, or simple prognosis, can be provided by monitoring NK cell numbers and optionally their state of activation. This will provide an extremely simple test. The methods described herein can be used to determine or estimate NK cell numbers in a sample taken from a patient. The numbers of NK cells and T cells with an NK-like activity (CD94/NKG2+) can be estimated by the use of antibodies against markers of NK cells (eg CD56 or CD16) or antibodies against T cell markers (eg CD3). The state of activation of the NK cells can be investigated by co-staining with antibodies to activation markers. Alternatively, the activation state of NK cells can be assessed in a functional assay *in vitro*. The NK cells are isolated from the sample and their cytolytic activity and/or ability to produce cytokines (eg interferon- γ and/or TNF- α) is assessed, either directly or after a short period in culture.

(ii) Infections

NK cell numbers may change during viral or other infections and knowing their numbers could be of great value for example in HIV

infected patients. It will be of particular interest to monitor NK cell numbers in cytomegalovirus (CMV) infections. CMV has sequences in its proteins that are capable of affecting HLA-E expression. More specifically, these CMV sequences induce cell surface expression of HLA-E in the virus host cell.

(iii) Pregnancy

There is interest in the role of NK cells in the placenta, in the prevention of rejection of the foetus. The invention provides a means to monitor NK cells in the placenta.

(iv) Transplantation

NK cells may be involved in transplant rejection and graft-versus-host disease (GVHD) after bone marrow transplantation. Monitoring of NK cells may be of value in patient management.

(v) Immunodeficiency

The diagnostic use of HLA-E can be extended to the detection of new immunodeficiency syndromes, either inherited or acquired, which exhibit lower or higher than normal NK cell levels. Some treatments may be toxic or stimulatory to NK cells.

(vi) Autoimmune Diseases

It will be useful to monitor NK cells in autoimmune diseases such as systemic lupus erythematosus, diabetes, thyroid diseases, vitiligo, rheumatoid arthritis etc.

(vii) Following Treatment

The invention also enables the monitoring of secondary effects of any treatment which could lead to up or downregulation of CD94/NKG2+ NK cells.

Although NK cells are specifically referred to in the examples of diseases and conditions above, monitoring of the T cell subset expressing receptors recognised by HLA-E is also included.

The invention also has a range of therapeutic applications.

Examples include the following:

(i) Enhancing NK Levels

The invention provides a method of selecting HLA-E binding
5 NK cells or T cells from a mixed cell population. The selected cells can be
expanded *in vitro* and returned to the patient. Such treatment may be
effective in some serious infections or cancers where a growth deficiency
of these cells is associated with poor prognosis.

(ii) Removing NK Cells

10 The invention provides methods and means for removal of
HLA-E binding NK cells and T cells, for example from bone marrow to be
used as donor bone marrow in transplantation. T cell depletion of bone
marrow using other techniques is already known and is effective. HLA-E
coupled to a toxin could also be used to destroy HLA-E binding cells *in*
15 *vivo*.

(iii) Expressing HLA-E at the surface of cells

The invention provides a method and means for inhibiting or
stimulating NK cell activity, by providing HLA-E at the surface of cells. NK
cells play a role in transplant rejection. Therefore, by ensuring that HLA-E
20 is expressed at the cell surface in a xenotransplant organ or tissue, the
likelihood of rejection will be reduced and rejection may be avoided
altogether.

Multimers of HLA-E in accordance with the invention can take
25 a variety of possible forms. The HLA-E molecules may be associated with
one another via a linker molecule. Alternatively or additionally the HLA-E
molecules may be attached to larger entities such as membrane structures,
for example liposomes. Suitable linker molecules include multivalent
attachment molecules such as avidin, streptavidin and extravidin each of
30 which has four binding sites for biotin. Thus, biotinylated HLA-E molecules

can be formed into multimer complexes of HLA-E having up to four HLA-E binding sites. The number of HLA-E molecules in the resulting complex will depend upon the ratio of HLA-E molecules to linker molecules used to make the complexes, and also on the presence or absence of any other
5 biotinylated molecules such as biotinylated signal moieties or toxic moieties. Preferred complexes are trimeric or tetrameric HLA-E complexes.

Other cross-linking techniques suitable for preparing HLA-E dimers or multimers include, for example, antibody cross-linking to produce
10 dimers, and techniques using cross-linking agents such as fluoregenic compounds or other chemical cross-linking agents.

Described herein in detail is a method of making HLA-E tetramers by biotinylating recombinant HLA-E heavy chains ($\alpha 1$, $\alpha 2$ and $\alpha 3$ domains) and constructing complexes by refolding the HLA-E heavy chains
15 *in vitro* with $\beta 2m$ molecules and with a synthetic leader peptide from HLA-B. In the method described, biotinylation sites are engineered into the C terminus of the HLA-E heavy chain and the recombinant HLA-E molecules are biotinylated by means of a suitable biotinylating enzyme. The multivalent linker molecule, in this case extravidin, is then added.
20 Variations of this tetramerisation method are envisaged. For example, the $\beta 2m$ could be chemically biotinylated at its seven lysine residues, four of which are in an appropriate position to allow complexing.

Whatever form of HLA-E is used, a suitable peptide for binding in the HLA-E peptide groove will be required. Tables 1 and 2 show
25 examples of possible peptides found at residues 3 to 11 of MHC class I leader sequences. The tables indicate which of these are found to bind to HLA-E *in vitro*. Other peptides which bind to HLA-E may be used, for example peptides from viral proteins which may be the same as or different to MHC class I leader sequence peptides. Although nonamer peptides are
30 usually the optimum size for binding to HLA-E, it is possible that peptides

which are slightly shorter or slightly longer, e.g. by one or two residues at one or both ends, will also work. A suitable assay for identifying synthetic peptides which bind to HLA-E is described in Braud et al 1997 *Eur. J. Immunol.* 27:1164-1169.

5 Labelling of the HLA-E may be carried out by any suitable method. Described herein is tetrameric HLA-E labelled with phycoerythrin via the extravidin used to form the tetrameric complex. A variety of other signal moieties may be employed at a variety of possible sites on the HLA-E monomer or multimer, and the HLA-E may be labelled before, during or
10 after multimerisation. Useful signal moieties commonly employed for labelling proteins include radioactive (e.g. ^{32}P or ^{33}P or ^{35}S), fluorescent (e.g. FITC) and enzymatic (e.g. horseradish peroxidase) labels. These and other detectable labels may be employed in the invention.

 HLA-E is also provided according to the invention linked to a
15 support. The HLA-E thus immobilised is particularly useful for capture purposes, that is for capturing and separating CD94/NKG2+ cells. A mixture containing or suspected of containing CD94/NKG2+ cells is brought into contact with the immobilised HLA-E under binding conditions and unbound material is removed. CD94/NKG2+ cells may then be
20 recovered. Examples of suitable solid supports include but are not limited to support bodies or particles which may be for example in the form of beads, wells, tubes and plates. The HLA-E may be immobilised by a variety of means well-known in the art and may be attached to the support by means of a suitable linker molecule.

25 HLA-E is also provided according to the invention attached to an effector agent such as a toxic moiety, the purpose of which may be the targeted depletion of CD94/NKG2+ cells in a mixed population of cells *in vitro* or *in vivo*. The toxic moiety is preferably a toxin which selectively kills the cells to which the HLA-E is bound. Suitable toxins can be derived from
30 natural sources such as plants (for example ricin, abrin) or bacteria (for

example diphtheria toxin, *Pseudomonas* exotoxin), or they may be synthetic or synthetic versions of naturally occurring toxins. In one particular example, the toxin is coupled to HLA-E by means of a linker molecule that introduces a disulphide bond between the HLA-E and the toxin (Vitetta et al, *Biol. Therapy of Cancer*, 1991, 482-495; Myers et al, *J. Immunol. Methods*, 1991, 131: 221-237). Thioether or other related compounds may be used to form non-reducible linkages. In another example, the toxin is expressed as a fusion protein with the HLA-E molecule, by splicing the gene encoding the toxin to the gene encoding the ligand to create a recombinant fusion protein (Pastan et al, *Annu. Rev. Biochem.*, 1992, 61: 331-354). Such fusion proteins may also include a biotinylation site to enable the creation of tetrameric or multimeric complexes of the toxin and HLA-E.

In general, the HLA-E described herein for use in detecting and separating and targeting toxins to CD94/NKG2+ cells will be a soluble HLA-E molecule. The term "soluble" is used herein in the manner in which it is conventionally used in the art in relation to cell surface receptors. A soluble form of a cell surface receptor is usually derived from the native form by deletion of the transmembrane domain. The protein may be truncated by removing both the cytoplasmic and the transmembrane domains, or there may be deletion of just the transmembrane domain with part or all of the cytoplasmic domain being retained. The important thing is that the desired extracellular function of the receptor is retained, which in this case is the CD94/NKG2-binding capability of the HLA-E binding domain. Wild type HLA-E may be modified to achieve the desired form by proteolytic cleavage, or by expressing a genetically engineered truncated or partially deleted form.

HLA-E for use in the invention which is present at surface of a cell on the other hand is preferably, although not necessarily, a membrane-bound form of HLA-E containing a transmembrane domain. It

may or not also have a cytoplasmic domain. In order to get HLA-E expressed at the surface of a cell which does not normally express HLA-E at its surface, it is necessary to provide a peptide which binds in the HLA-E peptide binding groove. The peptide may be derived either from an HLA leader sequence which is permissive for HLA-E expression, or it may be derived from another source which expresses peptides that bind to HLA-E and induce its expression. Such other sources include viruses which escape NK cell-mediated cytotoxicity by encoding a peptide which binds to HLA-E and induces its expression. For example, the human cytomegalovirus (HCMV) encodes a protein known as UL40 (Accession No. p16780) which possesses a peptide (VMAPRTLIL) capable of binding to HLA-E (see Table 1).

Thus, HLA-E expression at the surface of a non-human mammalian cell may be achieved for example by co-transfecting the cell with nucleic acid encoding HLA-E and another HLA class I which has a leader sequence peptide capable of binding to HLA-E. Alternatively the cell may be co-transfected with nucleic acids encoding HLA-E and UL40 of HCMV. The inventors have shown that when transfected with UL40, cells expressing HLA-E intracellularly are induced to express HLA-E at the cell surface. Instead of co-transfecting the cells with two different sequences, the cells can be transfected with a single vector containing both sequences, or more conveniently, with a chimeric nucleic acid which encodes a fusion protein of HLA-E with the peptide. This chimeric nucleic acid may be for example a recombinant nucleic acid encoding HLA-E, in which the leader sequence of HLA-B8 replaces the leader sequence of HLA-E. In another example, the nucleic acid encodes HLA-E linked to the peptide via a linker of sufficient length to allow the peptide to locate in the peptide-binding groove while also remaining covalently attached to the HLA-E molecule. In this instance, the peptide is not a part of the leader sequence which is cleaved off, and is instead linked to the mature HLA-E

molecule which is expressed at the cell surface. Advantageously, the peptide is linked to the HLA-E heavy chain via the HLA-E N-terminus (the α -1 domain). Such MHC-linked peptides are described in the published literature (see for example Mottez et al *J. Exp. Med.*, 1995, 181:493-502).

- 5 It may also be advantageous to express the HLA-E heavy chain and β 2m as a fusion protein. Such fusions are described in the literature (see for example Toshitani et al, *PNAS*, 1996, 93:236). Thus, the HLA-E-peptide molecule may be expressed from a single coding sequence.

- For the provision of HLA-E at the cell surface in organs or
10 tissues to be transplanted into another species, suitable transgenic animals can be produced. Techniques for generating transgenic animals such as transgenic pigs or rats or mice are well known in the art. For the purposes of the invention, nucleic acid material which expresses HLA-E and a suitable HLA-E binding peptide in the recipient organism is introduced into
15 cells of the organism at the appropriate early stage of development. Individual animals which express HLA-E at the surface of their cells are then selected. Organs or tissues from those animals provide xenogeneic material for transplantation.

- The absence of HLA-E in xenogeneic cells makes them
20 susceptible to NK cell-mediated lysis because their MHC class I molecules fail to be recognised by human killer cell inhibitory receptors. This is evident from inducing expression of HLA-Cw0301 on porcine endothelial cells, which protects the porcine cells against xenogeneic cytotoxicity mediated by NK cells expressing the inhibitory NK receptors binding to
25 HLA-Cw0301 (Seebach et al, *J. Immunol.*, 1997, 159:3655). There is evidence that NK cells play a role in the cellular immune response against xenografts (reviewed in Kaufman et al, *Ann. Rev. Immunol.*, 1995, 13:339 and Bach et al, *Immunol. Today*, 1996, 17:379). The endothelium is the first site of contact between a vascularized xenograft and the recipient

immune system. It has been shown that human NK cells adhere to the vascular endothelium and infiltrate into the xenogeneic organs (Kirk et al, *Transplantation*, 1993, 56:785; Inverardi, *Immunol. Rev.*, 1994 141:71) and that NK cells directly activate porcine endothelial cells (Goodman et al, *Transplantation*, 1996, 61:763). It has been observed that xenogeneic human anti-porcine cytotoxicity *in vitro* includes an important MHC unrestricted contribution from polyclonal NK populations (Inverardi, *Immunol. Rev.*, 1994, 141:71; Kirk et al, *Transplantation*, 1993, 55:294; Seebach et al, *Xenotransplantation*, 1996, 3:188). It has also been shown that transgenic mice expressing a killer cell inhibitory receptor from the immunoglobulin superfamily, CD158, which recognises HLA-Cw3, are prevented from rejecting H-2 mismatch bone marrow grafts which express the cognate MHC class I HLA-Cw3 allele (Cambiaggi et al, *PNAS*, 1997, 94(15):8088-92. Thus, an HLA-E transgenic animal can be used to provide organs which will not be prone to attack by human NK cells expressing CD94/NKG2A receptors.

Xenogeneic transplantation will also involve the use of other mechanisms and/or reagents for the purpose of improving transplant survival. In particular, immunosuppressive agents may be employed. Immunosuppressive drugs are commonly used in transplantation therapy.

Currently, the primary animal species proposed as sources of xenografts are pigs and baboons (and possibly cows). Examples of particular treatments currently under consideration are:

- treatment of Parkinson's disease by implantation of porcine foetal neuronal tissue;
- treatment of diabetes mellitus by implantation or infusion of encapsulated porcine pancreatic islet cells;
- treatment of hepatic failure by perfusion through or implantation of whole pigs livers;

(see Deacon, *Nat. Med.*, 1997, 3:350; Tibell, *Transplant. Proc.*, 1994,

26:762; Cramer, *Transplant. Proc.*, 1995, 27:80).

It will be evident that in addition to any special features such as the absence of transmembrane and/or cytoplasmic domains, or the presence of biotinylation sites required for multimerisation, the recombinant
5 HLA-E used in the invention may have other features which make it different to native HLA-E. For example, the recombinant HLA-E may have deletions or insertions or altered residues compared to native HLA-E, which result in improved properties such as enhanced binding capability or improved stability, for use in accordance with the invention. HLA-E having
10 improved stability at elevated temperatures, such as temperatures over 4°C and/or over room temperature and/or at or around 37°C, will be of particular interest.

HLA-E in recombinant form as described herein is also provided in formulations suitable for *in vivo* use. Such formulations
15 comprise a pharmaceutically acceptable diluent or carrier.

HLA-E itself exhibits very little polymorphism. The sequences for two different alleles of HLA-E can be found in the following data base locations: E*0101 at M20022 (arg in the residue at position 107); E*01031 at M32507 (glycine residue at position 107).

20 Some further, known techniques the principles of which may be additionally applied in the separation, identification or targeted killing methods of the invention are discussed below.

Some known separation methods will be adaptable for use in the separation or isolation of CD94/NKG2+ cells using HLA-E. For
25 example, T cell populations can be isolated by use of antibody-coated plates. The antibodies are specific for particular cell-surface markers. Cell separation can be a negative selection process or a positive selection process (Wysocki et al, 1978 *PNAS* 75:2840-2848). HLA-E coated plates may be used to separate CD94/NKG2+ cells.

30 Immunomagnetic purification of a T cell subpopulation can

also be realised using suitable antibodies coated on magnetic beads, in a negative or a positive selection process (Funderud et al 1987 in *Lymphocytes: A Practical Approach* Oxford University Press, New York 55-61). HLA-E-coated beads may be similarly employed for selection of

5 CD94/NKG2+ NK cells and T cells.

FACS (fluorescence activated cell sorting) techniques may also be employed. Cell sorting of fluorescence-labelled cells uses flow cytometry to monitor the expression of specific intracellular and cell surface molecules and sort cell populations (Fleisher et al 1988 *Cytometry* 9:309-

10 315).

Techniques which may be used in accordance with the invention for selective depletion or targeted killing of CD94/NKG2+ cells in a mixed cell population include antibody/complement-mediated cytotoxicity. Using a complement-fixing antibody, the cells expressing the marker

15 recognised by the antibody can be lysed in presence of complement (Bianco et al 1970 *J. Exp. Med.* 132:702-720). For example, an anti-HLA-E antibody may be employed to selectively destroy cells to which HLA-E is bound.

TABLE 1

Examples of peptides generated from MHC class I leader sequences
5 at residues 3-11

Leader sequence peptide (3-11) from MHC class I	Binding to HLA-E in vitro
-VMAPRTLVL	+
-VMAPRTLIL	+
-VMAPRTVLL	+
-VMAPRTLFL	+
-VMGPRTLVL	+/-
-VTAPRTVLL	-
-VTAPRTLIL	-
-VMPRTLIL	+
-VMEPRTLIL	-
-VMAPRALIL	-

[SEQ ID NOS: 1 - 11]

EXAMPLES

Example 1

Construction of HLA-E tetrameric complexes

HLA-E-tetrameric complexes were constructed by refolding
 5 recombinant HLA-E and β 2m molecules *in vitro* with a synthetic peptide
 (VMAPRTVLL) derived from residues 3-11 of the signal sequence of HLA-
 B*0801. A biotinylation site was engineered in the C terminus of the HLA-
 E heavy chain, allowing HLA-E/ β 2m/peptide complexes to be enzymatically
 biotinylated using *E.coli* BirA enzyme and conjugated with phycoerythrin
 10 (PE)-labelled Extravidin to create tetrameric complexes. HLA-A and -B
 tetrameric complexes have proved to be very efficient at specifically binding
 to T cell receptors on antigen-specific CD8⁺ T cells from peripheral blood *in*
vitro (Altman et al 1996 *Science* 274: 94-96).

Methods

HLA-E was cloned by RT-PCR with primers COO7 and
 COO6 from RNA extracted from monocytes of an HLA-E*0101
 homozygous individual. The N terminal nucleotide sequence was
 synonymously altered by PCR mutagenesis using the primers CO17 and
 20 COO6 to optimise protein expression from the pGMT7 vector in *E. coli*.
 The coding sequence for the extracellular portion of HLA-E (residues 1-
 276) was amplified using the primers CO17 and CO23 and recloned into a
 pGMT7 derivative to produce the expression plasmid COCO92 which
 contains the BirA recognition and biotinylation site in frame at the 3' end of
 25 the HLA-E heavy chain. Primers were:
 COO6 gtgggctaagcttacggctccatctcagggtgacgggctc [SEQ ID NO: 12]
 COO7 ctacgggcatatggtagatggaaccctcctttactctcc [SEQ ID NO: 13]
 CO17 ccgtacctcgagcatatgggttctcattctttaaataatttcatactctgtatctagaccggccg
 [SEQ ID NO: 14]
 30 CO23 tgggtgtctagaggatcctggctccatctcagggtgacgggctcg [SEQ ID NO: 15]

HLA-E tetrameric complexes were generated essentially as described (Altman et al 1996). Briefly, HLA-E and $\beta 2m$ proteins were over-expressed *in E. coli* strains BL21 (DE3) pLysS and XA90 respectively, purified from inclusion bodies, solubilised into a urea solution, then
5 refolded by dilution *in vitro* with a synthetic peptide (VMAPRTVLL) from HLA-B*0801 leader sequence (Research Genetics). HLA-E heavy chain/ $\beta 2m$ /peptide complexes were biotinylated with BirA enzyme, purified by FPLC and Mono-Q ion exchange chromatography, then complexed in a 4:1 molar ratio with Extravidin-PE (Sigma).

10

Example 2

Binding of HLA-E tetramers

Peripheral blood mononuclear cells (PBMC) from 9 normal donors were stained with HLA-E tetramer prepared as described in
15 Example 1 and compared to staining observed with an HLA-A2 tetramer refolded with Epstein Barr Virus (EBV) lytic cycle BMLF1 259-267 peptide epitope (Steven et al 1997 *J.Exp. Med.* 185: 1605-17). A high frequency of lymphoid cells were stained with the HLA-E tetramer (range 2 to 11%) (Fig. 1A), whereas the HLA-A2 tetramer generally stained 0 to 0.8% of the
20 lymphocytes in EBV-seropositive donors (Fig. 1C). By setting an electronic gate on the lymphocytes binding HLA-E tetramer, we observed that a large proportion were NK cells (typically 40 to 80 % CD3⁺, CD56⁺) but a significant subset were T cells (typically 15 to 50% CD3⁺), some of which were also expressing CD56 (Fig. 1B). About 2% of the lymphocytes
25 binding HLA-E tetramer were CD4⁺ T cells, and about 5 % were CD19⁺ B cells, but these could represent non-specific binding because of similar staining with the HLA-A2 tetramer (data not shown). The HLA-A2 tetramer did not bind to CD56⁺ cells but, in EBV-seropositive donors, bound to EBV specific CD3⁺, CD8⁺ T cells (Fig. 1D), confirming previous studies on the
30 specificity of MHC-tetrameric complexes for T cells bearing a specific T cell

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receptor (Altman et al 1996).

HLA-E tetramer staining was abolished when the PBMC and the tetramer were incubated in the presence of the antibody HP3D9 (Aramburu et al 1990 *Immunol.* 144: 3238-47) against CD94, an NK cell
5 receptor belonging to the C-type lectin superfamily (Chang et al 1995 *Eur J. Immunol.* 25: 2433-37) (Fig. 2A). As the antibody HP3D9 was diluted, the HLA-E tetramer staining was restored (data not shown). The interaction between HLA-E and CD94 was also confirmed by staining a number of well-characterised CD94⁺ NK clones with HLA-E tetramer and
10 demonstrating that another anti-CD94 mAb (DX22) (Phillips et al 1996 *Immunity* 5: 163-172) completely inhibited HLA-E tetramer binding (Fig. 2B, and data not shown). No staining with HLA-A2 tetramer was found on CD94⁺ NK clones (data not shown).

To characterise further the NK receptor interacting with HLA-
15 E, we stained P815 and 293T cells transfected with these receptors. No HLA-E tetramer staining was observed on P815 stably transfected with CD94 alone or NKG2B alone (Fig. 3A), nor on 293T transiently transfected with CD94 or NKG2A alone (data not shown). In contrast, HLA-E tetramer bound to 293T cells cotransfected with CD94 and NKG2A, CD94 and
20 NKG2B, or CD94 and NKG2C (Fig. 3B). Expression of the heterodimers on these transfectants was monitored using a polyclonal rabbit serum that reacts with CD94/NKG2A, NKG2B and NKG2C heterodimers (Lazetic et al 1996 *J. Immunol.* 157: 4741-45). This result was confirmed using mouse pre-B Ba/F3 cells stably transfected with CD94/NKG2C or NK clones
25 expressing the inhibitory receptor CD94/NKG2A (Fig 2B and data not shown). Carbohydrates on HLA-E are not necessary for binding, as the recombinant HLA-E used to make the tetramer was produced in *E. coli*. This is quite surprising given that both CD94 and NKG2 proteins are members of the C-type lectin superfamily. Carbohydrate residues may
30 form additional points of interaction increasing the affinity of binding.

We have also shown that HLA-E does not interact with other killer cell inhibitory cell receptors (KIR) as no staining with the HLA-E tetramer was observed on Ba/F3 cells transfected with KIR2DL1 (NKAT1 or p58), KIR2DL3 (NKAT2 or p58), KIR3DL1 (NKAT3 or p70), KIR3DL2 (NKAT4 or p70/140), KIR2DS2 (NKAT5 or p50), KIR2DL2 (NKAT6 or p58) or KIR2DS4 (NKAT8 or p50) (Lanier et al 1997 *Immunol. Rev.* 155: 145-154). Furthermore, staining of PBMC with HLA-E tetramer was not blocked by antibodies against any of these KIR receptors: EB6 (anti-KIR2DL1), GL183 (anti-KIR2DL3, -KIR2DS2, -KIR2DL2), DX9 (anti-KIR3DL1), or 5.133 (anti-KIR3DL1, -KIR3DL2) (data not shown). Thus, the CD94/NKG2 receptors appear to be unique and specific receptors for HLA-E recognition.

We have previously reported that HLA-E, like the mouse Qa-1 molecule (Aldrich et al 1994 *Cell* 79: 649-658; DeCloux et al 1997 *J. Immunol.* 158: 2183-2191; Cotterill et al 1997 *Eur. J. Immunol.* 27: 2123-2132) can bind signal sequence-derived peptides from MHC class I molecules in vitro (Braud et al 1997) and recently showed that HLA-E cell surface expression is regulated by the binding of such peptides (Braud et al 1998). Most HLA-A and HLA-C alleles possess a leader peptide 3-11 that binds to HLA-E whereas only a third of HLA-B alleles do. The remaining B alleles have a Threonine at position 2 in the peptide instead of a Methionine. This substitution at a primary anchor residue disrupted peptide binding to HLA-E as measured in an *in vitro* peptide binding assay previously described (Braud et al 1997) (Table 2). Transfection of MHC class I alleles which have a leader peptide capable of binding to HLA-E into HLA-A, -B, -C, -G negative 721.221 cells resulted in expression of the endogenous HLA-E on the cell surface of 721.221 cells. When the leader sequence peptide was not capable of binding, no such upregulation of HLA-E at the cell surface was observed.

It has been shown previously that NK cells expressing an

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inhibitory CD94/NKG2A receptor do not kill 721.221 cells transfected with certain HLA-A, -B, -C, or -G alleles, but are able to lyse these transfectants in the presence of neutralising anti-CD94 or anti class I antibodies (Phillips et al 1996 *Immunity* 5: 163-172; Sivori et al 1996 *Eur. J. Immunol.* 26:2487-2492; Sivori et al 1996 *Transplant* 28: 3199-3203). A striking correlation between the presence of an HLA class I leader sequence peptide capable of binding to HLA-E causing its surface expression and the specificity of the CD94/NKG2A inhibitory receptor is shown in Table 2. All the MHC class I alleles which, upon transfection, protect 721.221 cells from killing by CD94/NKG2A+ NK clones have a peptide capable of binding to HLA-E. Similarly, all HLA alleles incapable of protecting against these clones lack an HLA-E binding leader peptide. Together with the direct evidence for physical interaction between HLA-E and CD94/NKG2A, these results indicate that inhibition by the CD94/NKG2A receptor is mediated by recognition of HLA-E rather than a broad range of HLA-A, -B, and -C molecules. In further support of this, the HLA-A2 tetramer refolded around a Tax peptide epitope of human T-cell lymphotropic virus HTLV1 (Garboczi et al 1996 *Nature* 384: 134-141) did not bind to CD94/NKG2A transfectants or NK cells expressing CD94/NKG2 receptors, despite the fact that HLA-A2 has been shown to have a protective effect against CD94/NKG2A+ NK clones and HLA-A2 target cell protection can be reversed in the presence of anti-CD94 or anti-class I antibodies. Furthermore we confirmed, by immunoprecipitation that the anti-class I antibody DX17, which inhibits interactions between class I molecules and CD94/NKG2A, also recognises HLA-E.

It has recently been demonstrated that recognition of 721.221 target cells by CD94/NKG2A⁺ NK clones can be inhibited by transfection of HLA-G, another nonclassical class I molecule mainly expressed on trophoblast cells (Soderstrom et al 1997 *J. Immunol.* 159: 1072-1075;

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Perez Villar et al 1997 *J. Immunol.* 158: 5736-5743; Pende et al 1997 *Eur. J. Immunol.* 27: 1875-1880). However, HLA-G also possesses a leader sequence peptide capable of binding to HLA-E and 721.221-G transfectants express a significant level of HLA-E. Similarly, Reyburn et al 5 1997 (*Nature* 386: 514-517) recently reported that human cytomegalovirus encodes a viral protein (UL18), with similarity to MHC class I, that can protect 721.221 cells from NK cell lysis, possibly involving CD94 receptors. Whether these observations can be explained by binding of HLA-G or UL18 leader peptides to the endogenous HLA-E molecules in 721.221 is 10 under investigation.

HLA-E also binds to CD94/NKG2C which has been shown to activate cytolytic activity in NK cell transfectants (Houchins et al 1997 *J. Immunol.* 158: 3603-3609) indicating that HLA-E is involved in regulating NK cell-mediated cytotoxicity via both CD94/NKG2A and CD94/NKG2B 15 inhibitory NK cell receptors and CD94/NKG2C stimulatory NK cell receptors. Our present results demonstrate a novel role for a non-classical class I molecule HLA-E and identify its predominant receptor. It remains to be determined whether the strong preference of HLA-E for binding signal sequence-derived peptides is simply to permit expression of HLA-E or 20 whether it is implicit in recognition by CD94/NKG2 receptors.

Table 2

.221 cells transfected with HLA class I alleles	Inhibition of killing by CD94/NKG2A + NK clones*	Presence of leader sequence peptide capable of binding to HLA-E§	HLA leader sequence peptide (residues 3-11)	Concentration of peptide required to obtain 50% of binding to HLA-E†
.221	-	- ‡		
.221-A*0201	+	+	VMAPRTLVL	0.06 µM
.221-A*0211	+	+	VMAPRTLVL	0.06 µM
.221-A*2501	+	+	VMAPRTLVL	0.06 µM
.221-A*2403	+	+	VMAPRTLVL	0.06 µM
.221-A*3601	+	+	VMAPRTLLL	0.3 µM
.221-B*0702	+	+	VMAPRTVLL	0.06 µM
.221-Cw*0102	+	+	VMAPRTLIL	0.3 µM
.221-Cw*0401	+	+	VMAPRTLIL	0.3 µM
.221-Cw*0304	+	+	VMAPRTLIL	0.3 µM
.221-Cw*0801	+	+	VMAPRTLIL	0.3 µM
.221-G	+	+	VMAPRTLFL	0.3 µM
.221-B*1501	-	-	VTAPRTVLL	> 100 µM
.221-B*5101	-	-	VTAPRTVLL	> 100 µM
.221-B*5801	-	-	VTAPRTVLL	> 100 µM
.221-B*4601	-	-	VTAPRTVLL	> 100 µM
.221-B*5401	-	-	VTAPRTLLL	> 100 µM
.221-B*5501	-	-	VTAPRTLLL	> 100 µM

5 [SEQ ID NOS: 1 (4), 2, 3, 4 (4), 5, 7 (4), 8(2), respectively]

*Results published by Phillips *et al* 1996

† A peptide binding assay was developed in vitro. Results are expressed as a ratio of optical densities referred to as percentage of binding to HLA-E (Braud *et al* 1997)

10 ‡ The HLA-A, -B, -C, and -G negative .221 cells express HLA-E and HLA-F which have a shorter leader sequence and lack the appropriate peptide capable of binding to HLA-E.

§ The presence of a leader sequence peptide capable of binding to

HLA-E upregulates HLA-E surface expression as measured on .221 and .221 cells transfected with HLA-A or -B alleles using the antibody DT9 recognizing HLA-E and HLA-C alleles

5 Figure Legends for Example 2

Figure 1 HLA-E tetramer binds NK cells and a subset of T cells

Flow cytometry analysis on gated peripheral blood lymphocytes from normal EBV seropositive donor VB using (A) HLA-E tetramer refolded around the leader sequence peptide residues 3-11 from HLA-B*0801 or (C) HLA-A2 tetramer refolded around the Epstein Barr Virus (EBV) lytic cycle BMLF1 259-267 peptide epitope (Steven *et al* 1997). The phenotypes of (B) HLA-E tetramer or (D) HLA-A2 tetramer binding lymphocytes were further investigated in triple colour stains as indicated. Percentages in each quadrant are represented by the cross in the upper right. Within the total CD3-, CD56+ NK cell population, 10.3 % of cells bound HLA-E tetramer, and within the total CD3+ T cell population, 2.2 % of cells bound HLA-E tetramer. In contrast, less than 0.2 % of CD3-, CD56+ cells bound HLA-A2 tetramer, whereas 1 % of CD3+ T cells bound HLA-A2 tetramer.

Figure 2 HLA-E tetramer staining is inhibited by anti-CD94 antibodies

(A) Peripheral blood lymphocytes from normal donor SRJ were stained with the anti-CD94 antibody HP3D9 (Aramburu *et al* 1990) (1/50 dilution of ascites) followed by FITC-anti-mouse IgG (Fab')₂ (Sigma); HLA-E tetramer-PE alone; or HLA-E tetramer-PE in the presence of HP3D9 (1/50) which inhibited HLA-E tetramer staining.

(B) The NK cell line NKL (Robertson *et al* 1996 *Exp. Haematol.* 24: 406-415) expressing the NK receptor CD94/NKG2A but none of the KIR molecules was stained with the anti-CD94 antibody DX22 (Phillips *et al*

1996) (1mg) followed by PE-anti-mouse IgG; HLA-E tetramer-PE; or HLA-E tetramer-PE in the presence of 1 mg of DX22 antibody which inhibited HLA-E tetramer staining. Percentages in each quadrant are listed in the upper right. The HLA-A2 tetramer refolded around the HTLV1 Tax peptide
5 (Garbocz *et al* 1996) did not bind to NKL (data not shown).

Figure 3 HLA-E binds to NK cell CD94/NKG2A, CD94/NKG2B and CD94/NKG2C receptors but not to CD94 or NKG2 alone.

(A) P815 cells were stably transfected with pBJ-neo vector containing
10 human CD94 cDNA (Chang *et al* 1995) or NKG2B cDNA (Houchins *et al* 1991 *J. Exp. Med.* 173: 1017-20). Cells were stained with PE-control mouse IgG1 (cMIgG1) or IgG2b (cMIgG2b), anti-CD94 antibody DX22-PE, anti-NKG2A and B antibody DX20-PE, or HLA-E tetramer-PE. Neither P815 transfectant stained with HLA-E tetramer or HLA-A2 HTLV1 Tax
15 peptide tetramer. (B) 293T cells stably transfected with CD94 were transiently transfected with NKG2A, NKG2B, and NKG2C (Lazetic *et al* 1996). Flow cytometry staining was performed using rabbit preimmune serum (cRIgG) 1/500 final dilution or rabbit anti-CD94/NKG2 heterodimer serum (anti-CD94/NKG2) 1/500 final dilution, both followed by FITC- anti-
20 rabbit IgG, or with HLA-E tetramer-PE. No staining with HLA-A2-HTLV1 tax peptide tetramer was observed. Staining of 293T-CD94 cells cotransfected with a control plasmid were not stained by HLA-E tetramer or the rabbit anti-CD94/NKG2 serum (data not shown).

25 **Example 3**

Transfection of cells with HLA-E-binding leader sequences to enable HLA-E expression and protection against NK cell clones

Methods

Human NK-cell clones were established and cultured as
30 described (Litwin *et al* 1993 *J. Exp. Med.* 178: 1321-1336). Cytotoxicity

assays were performed as described (Phillips et al 1996 *Immunity* 5: 163-172). A chimeric cDNA containing the leader segment of HLA-G and the extracellular, transmembrane, and cytoplasmic domains of HLA-B*5801 was generated by PCR using the following oligonucleotide primers: sense
5 primer 1,5'-GCGTCTAGAATGGTGGTCATGGCACCCCGA-3' [SEQ ID NO: 16]; antisense primer 1,5'-CATGGAGTGGGAGCCGGCCAGGTCTCGGT-3' [SEQ ID NO: 17]; sense primer 2,5'-GGCTCCCACTCCATGAGGTAT-3' [SEQ ID NO: 18]; and antisense primer 2,5'-AAGCTTTCAAGCTGTGAGAGACA-3' [SEQ ID
10 NO: 19].

PCR was performed using a wild-type HLA-G cDNA as a template with primer set 1 and using wild-type HLA-B*5801 cDNA as a template with primer set 2. Products from these PCR reactions were mixed and used as templates for a subsequent reaction with sense primer
15 1 and antisense primer 2. The product was digested with *Xba*I and *Hind*III and ligated into the pBJneo vector.

A chimeric cDNA containing the leader segment of HLA-B*0702 and the extracellular, transmembrane and cytoplasmic domains of mouse CD80 (or B7-1) was generated by PCR using the
20 following oligonucleotide primers: sense primer 3,5'-ACCGAGACCTGGGCGTTGATGAACAACTG-3' [SEQ ID NO: 20]; antisense primer 3,5'-GCAAGCTTCTAAAGGAAGACGGTCTGTTC-3' [SEQ ID NO: 21]; sense primer 4,5'-GGGCGTCGACCCGGACTCAGAATCTCCTCAGACGCCGAG-3' [SEQ ID
25 NO: 22]; and antisense primer 4,5'-CAGTTGTTTCATCAACGGCCAGGTCTCGGT-3' [SEQ ID NO: 23].

PCR was performed using a wild-type mouse CD80 cDNA as a template with primer set 3 and using wild-type HLA-B*0702 cDNA as a template with primer set 4. Products from these PCR reactions were
30 mixed and used as templates for a subsequent reaction with sense primer

- 30 -

4 and antisense primer 3. The product was digested with *Sall* and *HindIII* and ligated into the pBJneo vector. PCR products were verified by sequencing. 721.221 B-lymphoblastoid cells were transfected with the wild-type and chimeric cDNAs and selected as described (Litwin et al
5 1993).

Results

To determine whether the presence of an HLA-E binding leader peptide that induces surface expression of HLA-E is enough to
10 provide protection against CD94/NKG2A⁺ NK-cell clones, a chimeric complementary DNA (GLS-B*5801) was generated. It contained the leader segment of HLA-G (from which a peptide can bind to HLA-E; Table 2) and the extracellular, transmembrane and cytoplasmic domains of HLA-B*5801 (an HLA molecule that is not implicated in recognition by
15 CD94/NKG2A receptors – Phillips et al 1996 *Immunity* 5: 163-172). Stable 721.221 cell line transfectants were selected and analysed for susceptibility to lysis by NK-cell clones expressing CD94/NKG2A receptors. As shown in Figure 4a, an NK-cell clone expressing a CD94/NKG2A receptor efficiently killed untransfected 721.221 cells as well
20 as 721.221 cells transfected with wild-type HLA-B*5801. However, protection against NK-cell-mediated lysis was conferred by expression of the chimeric GLS-B*5801 molecule but reversed in the presence of antibodies against either CD94 or HLA class I molecules. A chimeric cDNA (B7LS-mCD80) containing the leader segment of HLA-B*0702 (with
25 a peptide that can bind to HLA-E; Table 2) and the extracellular, transmembrane and cytoplasmic domains of mouse CD80 was transfected into 721.221 cells and tested for lysis by CD94/NKG2A⁺ NK-cell clones. CD80 is an adhesion cell surface molecule expressed on activated B and T cells and macrophages. In this experiment CD80 is used as an irrelevant
30 control molecule to show that only the leader sequence of MHC class I

molecules is necessary to upregulate HLA-E and induce a protective effect. There was less lysis of 721.221 cells expressing the B7LS-mCD80 molecule but not of cells expressing wild-type CD80, and protection was reversed by anti-CD94 but not control antibodies (Figure 4b). These results indicate that an HLA-E binding leader peptide alone is enough to protect 721.221 cells from lysis by NK-cell clones expressing inhibitory CD94/NKG2A-type receptors.

These results provide further confirmation of HLA-E as a ligand for CD94/NKG2A.

Figure Legends for Example 3

Figure 4 HLA-E mediates inhibition of NK cells through interaction with CD94/NKG2A.

(A) Lysis of 721.221 cells expressing HLA-B*5801, HLA-G or a chimeric molecule (GLS-B*5801) containing the HLA-G leader sequence and the extracellular, transmembrane, and cytoplasmic domains of HLA-B*5801 by a representative NK-cell clone expressing the CD94/NKG2A receptor.

Assays were performed at an effector to target ratio of 0.5:1, in the presence of control immunoglobulin (clg), anti-CD94 (DX22), or anti-HLA

class I (DX17) at $5\mu\text{g ml}^{-1}$. (B) Lysis of 721.221 cells expressing mouse CD80 or a chimeric molecule (B7LS-mCD80) containing the HLA-B*0702 leader sequence and the extracellular, transmembrane, and cytoplasmic domains of mouse CD80 by two representative NK-cell clones expressing the CD94/NKG2A receptor. Assays were performed at an effector-to-target ratio of 1:1 in the presence of control immunoglobulin (clg) or anti-CD94 (DX22) at $10\mu\text{g ml}^{-1}$.

Example 4

Isolation of CD94/NKG2+ cells by fluorescence activated cell sorting

Peripheral blood mononuclear cells (PBMC) were obtained

from venous blood which had been taken from donors into tubes containing Heparin. Briefly, blood samples were diluted 1:1 with serum free RPMI-1640 and 10 ml of diluted blood was laid onto a 5 ml Ficoll-Hypaque gradient. After a centrifugation at 1200 rpm for 30 minutes, the
5 PBMC at the interface were carefully removed and washed twice in RPMI. The first centrifugation was performed for 10 minutes at 2000 rpm and the second for 10 minutes at 1200 rpm to remove most platelets. PBMC were then diluted in RPMI and kept in sterile medium while processed.

Binding of HLA-E tetramer was monitored by flow cytometry
10 and cells were sorted. PBMC (5×10^6) were incubated for 15 minutes at 37°C followed by 15 minutes at 4°C with $12 \mu\text{l}$ of HLA-E tetramer labelled with phycoerythrin (PE). CD3 monoclonal antibody labelled with FITC was then added for another 15 minutes at 4°C . Cells were then washed twice and sorted on a FACScan, which measures fluorescent light emission and
15 separates distinct cell populations by electrostatic-deflection (electronic cell sorting). Single cells or subsets of cells stained by HLA-E tetramer were collected in sterile 96 well plates and put in culture. NK cells (CD3-, HLA-E tet+) and T cells (CD3+, HLA-E tet+) were grown in Yssel's medium (Yssel et al, 1984, *J. Immunol. Methods*, 72:2199) in the presence of irradiated
20 feeder cells (PBMC and JY BCL), $0.1 \mu\text{g/ml}$ of PHA, and 100 U/ml or 10 U/ml of IL-2 respectively. Autologous cells processed in this way are suitable for reinjection into patients. FACS techniques can also be used to count cells for quantitation purposes.

Suitable methods for culturing NK cells and clones are
25 described in Litwin et al, *J. Exp. Med.*, 1993, 178:1321-1336. Methods for maintaining T cells are described in Dunbar et al, *Current Biol.*, 1998, 8(7) 413 and Nixon et al, *Nature*, 1988. 336: 484-487.

Example 5Isolation of CD94/NKG2+ cells using HLA-E-coated beads

Cells expressing CD94/NKG2 receptors were isolated with HLA-E-streptavidin coated dynabeads. Dynabeads M-280 Streptavidin are magnetic beads coated with streptavidin. Soluble HLA-E was engineered with a biotinylation site for BirA enzyme at the C terminus of HLA-E heavy chain and refolded with $\beta 2$ microglobulin and a synthetic peptide derived from residues 3-11 of the signal sequence of some HLA molecules (described in Braud et al, 1998, *Nature*, 391:795). These HLA-E monomers were biotinylated using BirA enzyme and conjugated to Dynabeads M-280 using a standard protocol. Biotinylated HLA-E were incubated with PBS-washed Dynabeads M280 for 30 minutes at 4°C with bidirectional mixing (2 μ g HLA-E/ 10^7 Dynabeads). The beads were collected by placing the tube in a Dynal Magnetic Particle Concentrator (MPC) and the supernatant was removed. The beads were washed 5 times in the same way. HLA-E coated M-280 dynabeads were then mixed with isolated PBMC (obtained as described in Example 3) (10^7 beads/ml) and incubated for 20 minutes at 4°C with gentle rotation. The tube was placed in a Dynal MPC and left to rest for 2 minutes. The supernatant was removed and the cells attached to the beads washed 5 times. Cells were then grown as described in Example 3.

NB: To deplete PBMC of cells expressing receptors for HLA-E, the supernatant is kept and the beads discarded.

Example 6Targeted killing of NK cells

Recombinant biotinylated HLA-E is prepared as described in Example 1. A mixture containing biotinylated HLA-E and a biotinylated toxic agent such as the enzyme perforin in a molecular ratio of 3:1 is combined with extravidin to produce multimeric HLA-E linked to the toxic agent. A PBMC

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sample from a human donor is prepared according to standard techniques and contacted with the HLA-E reagent, resulting in killing of the CD94/NKG2+ cells present in the sample. CD94/NKG2 negative cells are recovered.

5

Example 7

Xenotransplantation

A recombinant DNA expressing HLA-E in which the leader sequence of HLA-E was replaced by the leader sequence of HLA-B8 was generated. This chimeric cDNA contains the leader sequence of HLA-B, and the extracellular ($\alpha 1$, $\alpha 2$ and $\alpha 3$), the transmembrane and the cytoplasmic domains of HLA-E. It was generated using the following oligonucleotide primers:

sense primer A:

15

5'-

CTCGGCGGCCCTGGCCCTGACCGAGACCTGGGCGGGCTCCCACTCC
TTG-3' [SEQ ID NO: 24]

antisense primer B:

20

5'-TTCTGTCTAGATTACAAGCTGTGAGACTCAGACCCCTG-3' [SEQ ID
NO: 25]

sense primer C:

5'-

CTGACCGAATTCGCCGCCACCATGCTGGTCATGGCGCCCCGAACCGT
CCTCCTGCTGCTCTCGGCGGCCCTGGCC-3' [SEQ ID NO: 26]

25

The PCR was performed using the cDNA of HLA-E as a template with primers A and B. The product from that PCR was then used as a template for a subsequent reaction with primers B and C. The last product was digested with EcoR1 and XbaI and ligated into the expression vector pcDNA3.

30

Transgenic animals are then produced as follows. Females

- 35 -

are superovulated, mated to fertile males and sacrificed the following day. Zygotes with two pronuclei are recovered and one of the pronuclei is microinjected with the DNA expressing the HLA-E-HLA-B leader sequence construct. Surviving embryos are reimplanted into pseudopregnant foster
5 females and DNA samples from new borns are evaluated for the presence of the foreign gene (HLA-E construct). These techniques are described in detail in the literature (eg Guide to techniques in mouse development, P. Wassarman and M DePamphilis, Methods in Enzymology (Academic Press), Section X: Transgenic animals: pronuclear injection (p747-802)
10 and Section XI: Transgenic animals: embryonic stem cells and gene targeting (p803-932)).

Example 8

Stable transfection of CD94 and NKG2 genes into mammalian cells

15 Mouse cells (P815, L cells) were sequentially transfected by electroporation or calcium phosphate DNA precipitation respectively, with a mammalian expression vector pcDNA3 (neomycin resistance gene) containing CD94 cDNA and either NKG2A or NKG2C with DAP12. NKG2A and NKG2C cDNA was cloned into the expression vector
20 pcDNA3.1/hygro vector (containing the hygromycin resistance gene) and DAP12 was cloned into the expression vector pcDNA3.1/zeo (zeomycin resistance). P815 cells were electroporated with 500 μ F, 0.25 volt, and selection (G418 and hygromycin and zeomycin) was added 2 days later. Cells expressing a high level of receptors were sorted by flow cytometry. L
25 cells were transfected by calcium phosphate DNA precipitation and selected in the presence of G418, hygromycin and zeomycin. Transfectants were cloned by limiting dilution and cell surface expression of the CD94/NKG2 receptors was monitored using specific antibodies.

The stable transfectants are useful for the identification of
30 antibodies or other agents that interfere with HLA-E binding to

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CD94/NKG2. Agents which specifically interfere with HLA-E binding to either inhibitory CD94/NKG2 receptors (e.g. CD94/NKG2A) or stimulatory CD94/NKG2 receptors (e.g. CD94/NKG2C) can be identified by performing binding assays using two different transfectants. A list of known antibodies
5 which interfere with HLA-E binding to CD94/NKG2 is given below:

Antibodies which block the interaction between HLA-E and CD94/NKG2 receptors:

1 - Anti-HLA-E: - 3D12 (Lee et al, 1998, *J. Immunol.* 160: 4951

2 - Anti-CD94:

10 - HP3D9 (Perez-Villar et al, 1995, *J. Immunol.* 154: 5779) commercialised by Pharmingen

- HP-3B1 (Aramburu et al, 1990, *J. Immunol.* 144: 3238) commercialised by Immunotech

15 - X1A85 (Sivori et al, 1996, *Eur. J. Immunol.* 26: 2487)

- DX22 (Phillips et al, 1996, *Immunity*, 5: 163) DNAX

3. Anti-NKG2A:

- Z199 (Carreto et al, 1997, *Eur. J. Immunol.* 27:563) commercialised by Immunotech

20 - Z270 (Sivori et al, 1996, *Eur. J. Immunol.* 26: 2487)

The techniques described in this example may also be used to transfect mammalian cells such as murine L cells with nucleic acids encoding HLA-E as described herein.

CLAIMS

1. A method of identifying the presence of CD94/NKG2+ NK
5 cells and T cells in a sample, which method comprises contacting the sample with HLA-E under binding conditions and detecting binding of HLA-E to the cells.
2. The method according to claim 1, wherein the HLA-E is labeled with a signal moiety and bound HLA-E is detected
- 10 3. A method of selecting CD94/NKG+ cells from a sample, which method comprises contacting the sample with HLA-E under binding conditions and separating cells bound to the HLA-E from the sample.
4. The method according to claim 3, wherein the HLA-E is immobilised on a support.
- 15 5. A method of killing or inactivating CD94/NKG2+ cells, which method comprises contacting the cells with HLA-E under binding conditions and carrying out targeted killing on the bound cells.
6. The method according to claim 5, wherein the HLA-E is attached to an effector agent.
- 20 7. The method according to claim 6, wherein the effector agent is a toxic moiety which kills or inactivates the bound cells.
8. The method according to any one of claims 5 to 7, wherein the CD94/NKG2+ cells are provided in a mixed cell population and the non-CD94/NKG2+ cells are recovered.
- 25 9. A method of modifying NK cell activity against a potential target cell, which method comprises expressing HLA-E at the target cell surface.
10. The method according to claim 9, wherein the HLA-E is expressed by a heterologous DNA stably integrated into the cell.

11. The method according to claim 10, wherein the cell does not normally express HLA-E.

12. The method according to claim 11, wherein the cell is a non-human mammalian cell.

5 13. The method according to any one of claims 9 to 12, wherein the cell is present in a group of cells in a xenogeneic organ or tissue.

14. CD94/NKG2+ cells isolated by the method according to claim 3 or claim 4.

10 15. A population of cells depleted of CD94/NKG2+ cells by the method according to claim 8.

16. A therapeutic method comprising introducing an effective quantity of cells according to claim 14 or claim 15 into a patient.

17. A method comprising removing a sample of cells from a patient, isolating from the sample a population of CD94/NKG2+ cells or a
15 population of CD94/NKG2 depleted cells, by the method according to claim 3 or claim 4, or claim 8, and reintroducing the isolated population of cells into the patient.

18. A non-human mammalian cell which expresses HLA-E at the cell surface by virtue of a nucleic acid encoding HLA-E integrated into the
20 genome of the cell.

19. A non-human mammal comprising cells according to claim 18.

20. A method of testing a compound for biological activity, which method comprises:

- 25 (i) providing cells expressing CD94/NKG2 receptors at the cell surface;
- (ii) contacting the cells with HLA-E in the presence of the test compound; and
- (iii) determining whether the presence of the compound affects
30 the binding of HLA-E to the cells.

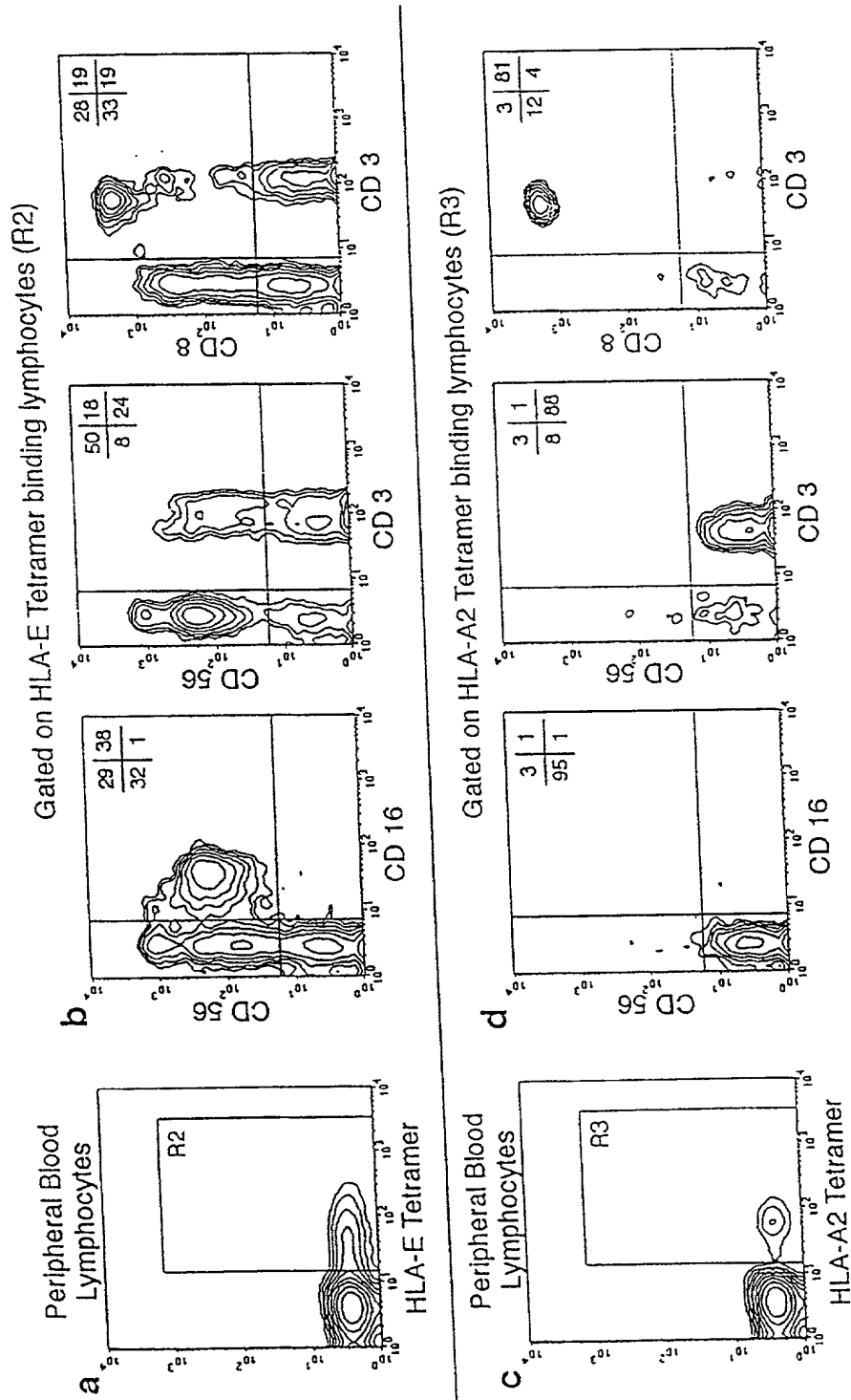
21. The method according to claim 20, wherein the CD94/NKG2 receptors are inhibitory NK cell receptors such as CD94/NKG2A receptors.
22. The method according to claim 20, wherein the CD94/NKG2A receptors are stimulatory NK cell receptors such as CD94/NKG2C
5 receptors.
23. Compounds identified by the method according to any one of claims 20 to 22, as affecting the binding of HLA-E to CD94/NKG2 receptors.
24. A multimer of HLA-E comprising two or more HLA-E
10 molecules, said multimer having enhanced binding capability compared to non-multimeric HLA-E, optionally labelled with a signal moiety.
25. The multimer according to claim 24, comprising recombinant HLA-E molecules attached via a linker molecule.
26. The multimer according to claim 25, wherein the HLA-E
15 molecules are biotinylated and attached via a linker molecule such as streptavidin, avidin or extravidin.
27. HLA-E coupled to a toxic agent.
28. The HLA-E according to claim 27, wherein the HLA-E is in the form of a multimer of HLA-E according to any one of claims 24 to 26.
- 20 29. HLA-E immobilised on a support.

ABSTRACT

The invention relates to a method of causing interaction of CD94/NKG2+ cells, which method comprises contacting the cells with recombinant HLA-E under binding conditions. The HLA-E property of binding to CD94/NKG2 receptors on NK cells and a subset of CD8+ T cells is useful for targeting CD94/NKG2+ cells for a variety of purposes such as identification. isolation, killing or inactivation.

5

Figure 1



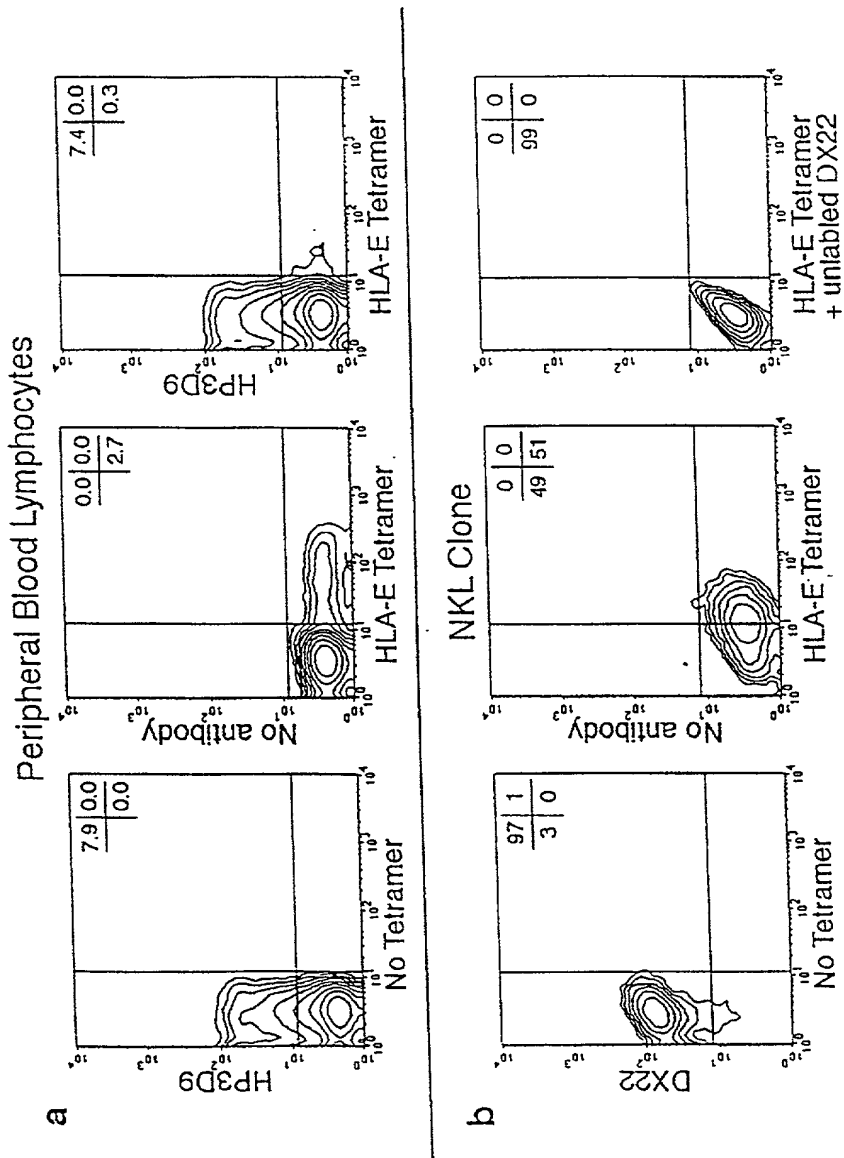
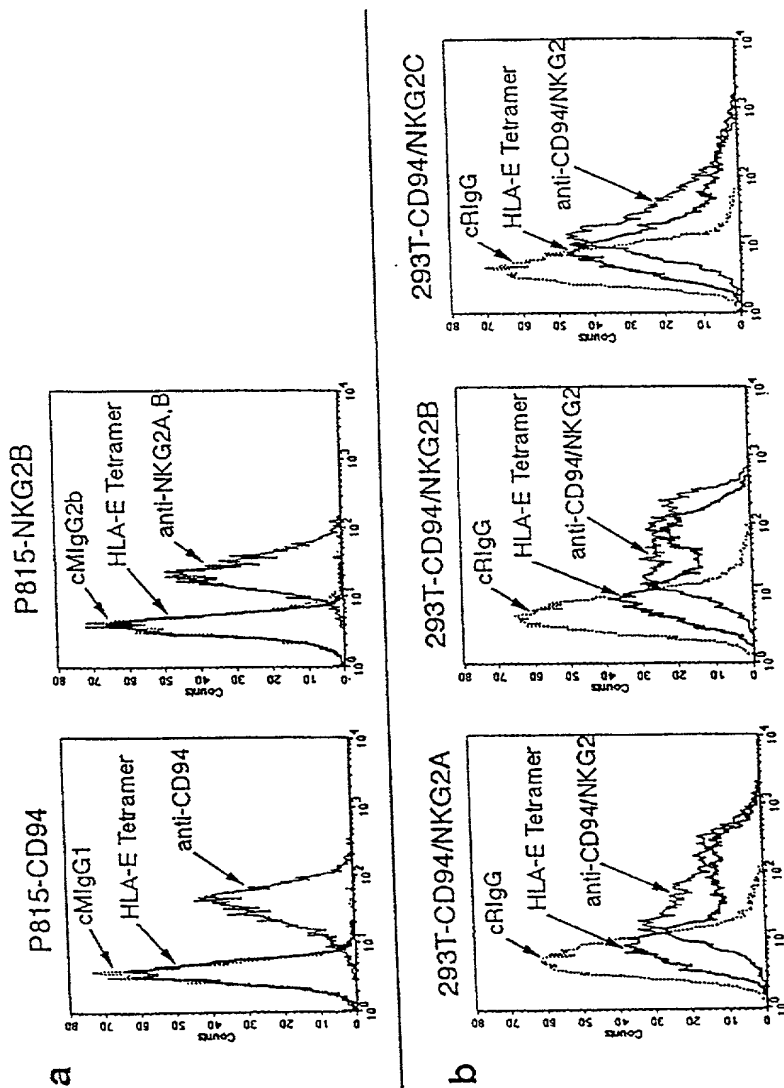
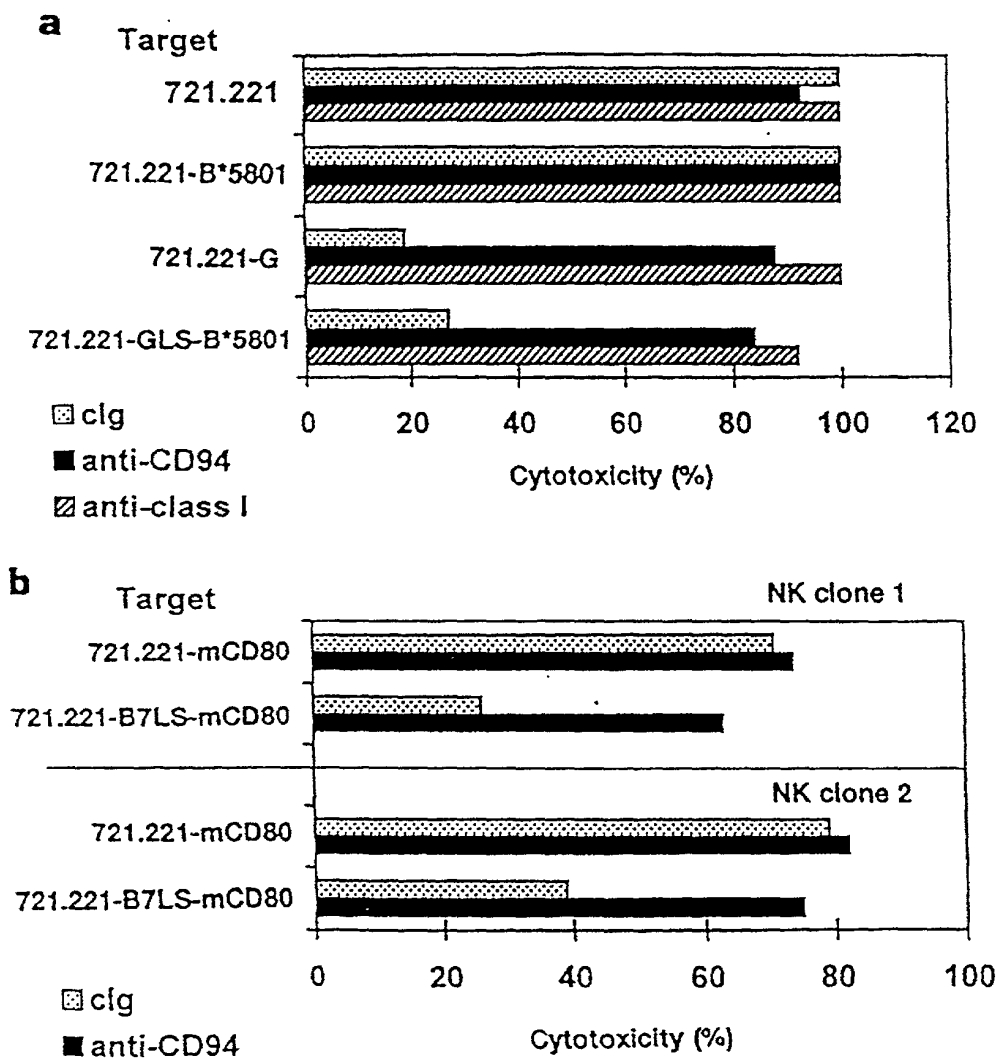


Figure 3



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Figure 4



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	Application Number	Not Yet Known
	Filing Date	Not Yet Known
	Group Art Unit	Not Yet Known
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HLA-E BINDING

the specification of which (Title of the Invention)

☐ is attached hereto OR ☒ was filed on (MM/DD/YYYY) 12/04/1998 as United States Application Number or PCT International

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Christopher A				O'Callaghan			
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City		State	CA	ZIP	91106	Country	USA

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DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 2 of 2

Name of Additional Joint Inventor, if any:					<input type="checkbox"/> A petition has been filed for this unsigned inventor				
Given Name (first and middle [if any])					Family Name or Surname				
Andrew J.					McMichael				
Inventor's Signature					Date				
Residence: City		State		Country		Citizenship		British	
Post Office Address									
Post Office Address									
City		State		ZIP		Country			
Name of Additional Joint Inventor, if any:					<input type="checkbox"/> A petition has been filed for this unsigned inventor				
Given Name (first and middle [if any])					Family Name or Surname				
Inventor's Signature					Date				
Residence: City		State		Country		Citizenship			
Post Office Address									
Post Office Address									
City		State		ZIP		Country			
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Inventor's Signature					Date				
Residence: City		State		Country		Citizenship			
Post Office Address									
Post Office Address									
City		State		ZIP		Country			

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☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

Family Name or Surname

Andrew J.

McMichael

Inventor's
Signature

Date

Residence: City

Beckley

State

Oxford

Country

UK

Citizenship

British

Post Office Address

Midsummer Cottage, Church Street, Beckley, Oxford, OX39UT, UK

Post Office Address

City

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)	Attorney Docket Number	SHP-PT059
	First Named Inventor	Braud et al.
	COMPLETE IF KNOWN	
	Application Number	Not Yet Known
	Filing Date	Not Yet Known
	Group Art Unit	Not Yet Known
<input type="checkbox"/> Declaration Submitted with Initial Filing	OR	<input checked="" type="checkbox"/> Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)
Examiner Name	Not Yet Known	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

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the specification of which (Title of the Invention)

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) 12/04/1998 as United States Application Number or PCT International Application Number PCT/GB98/03686 and was amended on (MM/DD/YYYY) 06/01/2000 (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
9725764.6	Great Britain	12/04/1997	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below

Application Number(s)	Filing Date (MM/DD/YYYY)

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[Page 1 of 2]

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	First Named Inventor	Braud et al.
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(Title of the Invention)

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			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

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I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below

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[Page 1 of 2]

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DECLARATION — Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
PCT/GB98/03686	12/04/1998	

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith

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OR

☒ Registered practitioner(s) name/registration number listed below

Place Customer
Number Bar Code
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Name	Registration Number	Name	Registration Number
Alfred Stapler	16,675	Glenn M. Massina	40,081
Anthony S. Volpe	28,377	Jeffrey M. Glabicki	42,584
C. Frederick Koenig III	29,662	Kao H. Lu	43,761
Randolph J. Huis	34,626		
Gerald B. Halt, Jr.	37,633		

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☐ Customer Number or Bar Code Label

OR ☒ Correspondence address below

Name	C. Frederick Koenig III, Esquire Volpe and Koenig, P.C.				
Address	Suite 400, One Penn Center				
Address	1617 John F. Kennedy Blvd.				
City	Philadelphia	State	PA	ZIP	19103
Country	U.S.A.	Telephone	(215) 568-6400		Fax (215) 568-6499

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon

Name of Sole or First Inventor:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle (if any))				Family Name or Surname			
Veronique M.				Braud			
Inventor's Signature						Date	
Residence: City		State		Country		Citizenship	French
Post Office Address							
Post Office Address							
City		State		ZIP		Country	

☒ Additional inventors are being named on the 2 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
PCT/GB98/03686	12/04/1998	

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Name of Sole or First Inventor:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle (if any)) <u>Veronique M.</u>		Family Name or Surname <u>Braud</u>	
Inventor's Signature <u>[Signature]</u>	Date <u>3/08/00</u>		
Residence: City <u>Shrivenham</u>	State <u>GB</u>	Country <u>UK</u>	Citizenship <u>French</u>
Home Post Office Address <u>32 Hazells Lane Shrivenham SN6 8DS</u>			
Work Post Office Address <u>Institute of Molecular Medicine John Radcliffe Hospital Headington</u>			
City <u>Oxford</u>	State	ZIP <u>OX3 9DS</u>	Country <u>UK</u>

☒ Additional inventors are being named on the 2 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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DECLARATION**ADDITIONAL INVENTOR(S)
Supplemental Sheet**Page 2 of 2

Name of Additional Joint Inventor, if any:				<input type="checkbox"/> A petition has been filed for this unsigned inventor			
Given Name (first and middle [if any])				Family Name or Surname			
Andrew J.				McMichael			
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**DECLARATION FOR UTILITY OR
DESIGN
PATENT APPLICATION
(37 CFR 1.63)**

☐ Declaration Submitted with Initial Filing OR ☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number SHP-PT059

First Named Inventor Braud et al.

COMPLETE IF KNOWN

Application Number Not Yet Known

Filing Date Not Yet Known

Group Art Unit Not Yet Known

Examiner Name Not Yet Known

As a below named inventor, I hereby declare that:

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the specification of which

(Title of the Invention)

☐ is attached hereto
OR

☒ was filed on (MM/DD/YYYY) 12/04/1998 as United States Application Number or PCT International

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			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

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I hereby claim the benefit under 35 U.S.C. 119(a) of any United States provisional application(s) listed below

Application Number(s)	Filing Date (MM/DD/YYYY)	<input type="checkbox"/> Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

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Veronique M.		Braud	
Inventor's Signature		Date	
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		Country	
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	Filing Date	Not Yet Known
	Group Art Unit	Not Yet Known
Examiner Name	Not Yet Known	

☐ Declaration Submitted with Initial Filing OR ☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

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the specification of which (Title of the Invention)

☐ is attached hereto
OR
☒ was filed on (MM/DD/YYYY) 12/04/1998 as United States Application Number or PCT International

Application Number PCT/GB98/03686 and was amended on (MM/DD/YYYY) 06/01/2000 (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
9725764.6	Great Britain	12/04/1997	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input checked="" type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below

Application Number(s)	Filing Date (MM/DD/YYYY)

☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

[Page 1 of 2]

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DECLARATION — Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
PCT/GB98/03686	12/04/1998	

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith

☐ Customer Number

OR

☒ Registered practitioner(s) name/registration number listed below

Place Customer
Number Bar Code
Label here

Name	Registration Number	Name	Registration Number
Alfred Stapler	16,675	Glenn M. Massina	40,081
Anthony S. Volpe	28,377	Jeffrey M. Glabicki	42,584
C. Frederick Koenig III	29,662	Kao H. Lu	43,761
Randolph J. Huis	34,626		
Gerald B. Halt, Jr.	37,633		

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto

Direct all correspondence to: ☐ Customer Number or Bar Code Label

OR ☒ Correspondence address below

Name	C. Frederick Koenig III, Esquire Volpe and Koenig, P.C.				
Address	Suite 400, One Penn Center				
Address	1617 John F. Kennedy Blvd.				
City	Philadelphia	State	PA	ZIP	19103
Country	U.S.A.	Telephone	(215) 568-6400	Fax	(215) 568-6499

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon

Name of Sole or First Inventor:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle (if any))		Family Name or Surname	
Veronique M.		Braud	
Inventor's Signature		Date	
Residence: City	State	Country	Citizenship
			French
Post Office Address			
Post Office Address			
City	State	ZIP	Country

☒ Additional inventors are being named on the 2 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 2 of 2

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

Family Name or Surname

Andrew J.

McMichael

Inventor's
Signature

Date

Residence: City

State

Country

Citizenship

British

Post Office Address

Post Office Address

City

State

ZIP

Country

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**DECLARATION FOR UTILITY OR
DESIGN
PATENT APPLICATION
(37 CFR 1.63)**

☐ Declaration Submitted with Initial Filing OR ☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number SHP-PT059

First Named Inventor Braud et al.

COMPLETE IF KNOWN

Application Number Not Yet Known

Filing Date Not Yet Known

Group Art Unit Not Yet Known

Examiner Name Not Yet Known

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06/01/2000

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			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

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OR

☒ Registered practitioner(s) name/registration number listed below

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Anthony S. Volpe	28,377	Jeffrey M. Glabicki	42,584
C. Frederick Koenig III	29,862	Kao H. Lu	43,761
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Gerald B. Halt, Jr.	37,633		

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City	Philadelphia	State	PA	ZIP	19103
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Given Name (first and middle (if any))		Family Name or Surname	
Veronique M.		Braud	
Inventor's Signature			Date
Residence: City	State	Country	Citizenship
			French
Post Office Address			
Post Office Address			
City	State	ZIP	Country

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DECLARATION

ADDITIONAL INVENTOR(S)

Supplemental Sheet

Page 1 of 2

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

Family Name or Surname

David S.J.

Allan

Inventor's
Signature

Date

Residence: City

State

Country

Citizenship

Canada

Post Office Address

Post Office Address

City

State

ZIP

Country

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

Family Name or Surname

Graham S.

Ogg

Inventor's
Signature

Date

Residence: City

State

Country

Citizenship

British

Post Office Address

Post Office Address

City

State

ZIP

Country

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

Family Name or Surname

Christopher A.

O'Callaghan

Inventor's
Signature

Date

Residence: City

State

Country

Citizenship

British

Post Office Address

Post Office Address

City

State

ZIP

Country

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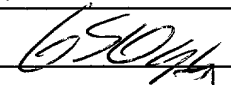
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DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 1 of 2

Name of Additional Joint Inventor, if any:						<input type="checkbox"/> A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any])				Family Name or Surname			
David S.J.				Allan			
Inventor's Signature					Date		
Residence: City		State		Country		Citizenship	
						Canada	
Post Office Address							
Post Office Address							
City		State		ZIP		Country	
Name of Additional Joint Inventor, if any:						<input type="checkbox"/> A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any])				Family Name or Surname			
Graham S.				Ogg			
Inventor's Signature					Date		2/18/00
Residence: City		State		Country		Citizenship	
Oxford		Oxon		UK		British	
Post Office Address							
19 St Christopher's Place							
Post Office Address							
CBX							
City		State		ZIP		Country	
Oxford		Oxon		OX2 4HS		UK	
Name of Additional Joint Inventor, if any:						<input type="checkbox"/> A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any])				Family Name or Surname			
Christopher A.				O'Callaghan			
Inventor's Signature					Date		
Residence: City		State		Country		Citizenship	
						British	
Post Office Address							
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